**Reviewer 1 Comments:**

1) The title can be revised as "MALDI-Mass Spectrometric Imaging for Metabolites Investigation on Medicago truncatula Root Nodules", since the MALDI-MS imaging as an analytical method is the key point in this paper while the sample can be any tissues besides Medicago truncatula Root Nodules.

**Response:** The title has been changed to “MALDI-Mass Spectrometric Imaging for Investigation of Metabolites in *Medicago truncatula* Root Nodules”  
  
  
2) Please specify the "High Resolution LC-MS" used in this study.

**Response:** The high resolution MALDI and LC- MS instruments used for accurate mass measurements in this study are now listed in the Material/Equipment table.

3) The sample preparation procedure for LC-MS analysis was missing.

**Response:** Since the manuscript focuses on MALDI-MSI and LC-MS is just supplemental and there are many different LC-MS sample preparation procedures depending on the analyte of interest, a detailed LC-MS description was omitted.

4) Page 4, line 151, please provide the start-up instructions, an online URL link to the instructions will work.

**Response:** The instructions were intentionally left vague as to not promote one type of automatic sprayer over any other system. The instructions will be clearly given and explained to the lab when the sprayer instrument is installed.

5) Page 5, line 194, "the smallest laser diameter", please specify the laser diameter.

**Response:** The laser diameters have been included in the description.

6) Page 5, line 207, please specify the "commercial software" used for image generation. Page 6, line 211, please specify the "instrument specific software" used for metabolite identification.  
  
*Editorial Comment: Please include this information in the Materials/Equipment table and not in the manuscript text as JoVE is unable to publish manuscripts containing commercial sounding language. You may refer the reader to the Materials/Equipment table in this step.*

**Response:** The vendor specific software has been listed in the Materials/Equipment Table and referred to in the text.  
  
7) Page 4, line 140, "DHB matrix solution (150 mg/mL in 50% methanol/0.1% TFA v/v)", line 154, "using 40 mg/mL (in 50% methanol/0.1% TFA v/v) DHB as the matrix". DHB matrix solutions with different concentrations were used for airbrush application and automatic sprayer application, respectively. Please elaborate the consequence in MALDI imaging by using different matrix solutions for matrix application.

**Response:** The particular sprayer system used here heats the nozzle for faster evaporation of the solvent. As the solvent evaporates, the concentration of the matrix increases quickly. The matrix applied to the sample with the airbrush and the automatic sprayer has comparable concentrations.

This information has been added to the matrix sprayer section.

8) Fig. 1, "Array of Spectra" is too blurry.

**Response:** The “Array of Spectra” has been fixed.

9) Fig. 4b, what are these square shapes in right up corners?

**Response:** The square shapes are the matrix only areas that were imaged. This has been specified more clearly now in the description of Figure 4b on page 7.

10) Please provide the optical image of the tested root nodule, perhaps in Fig. 4. Please add a scale bar of intensity in Fig. 4.

**Response:** A scale bar has been added to Figure 4.

A new figure (Figure 2) has been added showing an optical image of a root nodule section.

Figures have been re-numbered.

11) Fig. 4b shows the ion maps of matrix related compounds, which are not evenly distributed over the tested tissue. Does this mean the matrix was not evenly sprayed on the tissue section before imaging test?

**Response:** This means that there are different ionization efficiencies of the matrix on tissue vs. on the glass slide. The matrix was evenly sprayed.

12) Fig. 5, the x-axis was missing. What is the peak with an open circle in the bottom left corner?

**Response:** The x-axis has been added and the open circle has been removed.

13) Table 2, the mass error for the compounds detected in negative ion mode were too big, please elaborate.

**Response:** This is published work. The mass error was within acceptable limits for the instruments being used.  
  
 **Reviewer 2 Comments:**

Since a major focus of this paper is demonstration of 3 different matrix application techniques, a more thorough description of the components necessary for setting up each different application apparatus should be discussed. For instance: (1) airbrush application: what kind of airbrush is appropriate? Model # of one that is currently being used? Is this performed in a fume hood? (2) automatic sprayer - what sprayer system is being described? (3) sublimation: is this commercially available or needs to be assembled? specs? What vacuum settings / pump is used? 

**Response:** Matrix application apparatuses were referred to generically in the text of the manuscript, following editor instructions. The brand and catalog numbers for all equipment used is now listed in the Material/Equipment table. More information was added for the airbrush and sublimation procedure sections.

*Editorial Comment: Please remember to include all commercial language (brand and model #s) in the Materials/Equipment Table and refer the reader to the Table in the protocol section.*

**Response:** The Materials/Equipment table has been updated to include all brands and catalog numbers. The table has now been referred to throughout the protocol section.